

## AN INSIGHT TO RNA INTERFERENCE BASED GENE SILENCING

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**ABSTRACT**

The discovery of RNA interference (RNAi) is among the most significant biomedical breakthroughs in recent history. Multiple classes of small RNA, including small-interfering RNA (siRNA) and micro-RNA (miRNA) play important roles in many fundamental biological and disease processes. RNA interference, triggered by double-stranded RNA molecules, was initially recognized as a handy tool to reduce gene expression but now it is recognized as a mechanism for cellular protection and cleansing. It defends the genome against molecular parasites such as viruses and transposons, while removing abundant but aberrant nonfunctional messenger RNAs. Nonetheless, these new pools of knowledge have opened up avenues for unraveling the finer details of the small RNA mediated pathways. In this paper, we discuss the molecular aspects in biomedical research of RNA interference and its applications.

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**INTRODUCTION**

The studies on RNA led to the discovery of different classes of RNA's like, messenger RNA (mRNA), transfer RNA (tRNA), ribosomal RNA (rRNA) and small nuclear RNA (snRNA). Each of the different types of RNA are encoded by their own specific gene and they having various important roles in our body such as mRNA encodes amino acid sequence of various protein molecules, tRNA helps in bringing different amino acids to the ribosomes during the process of translation, rRNA along with various ribosomal proteins form ribosomes, snRNA which are only found in eukaryotes and help in RNA processing. Apart from these, the discovery of an independent novel class of RNA molecules (miRNA and siRNA) was made which were comparatively small (~19-30 nts), and were initially thought to be the degradation products of larger RNA molecules. It is these small RNAs which are now considered to regulate many cellular processes in the eukaryotes like replication, transcription, translation, chromosome structure, RNA editing. RNA interference (RNAi) is a novel gene regulatory mechanism that regulates a number of processes within living cells. It limits the transcript level by either suppressing transcription, transcriptional gene silencing (TGS) or by activating a sequence specific

RNA degradation process called post transcriptional gene silencing (PTGS/RNAi) (1). This phenomenon was first observed by Fire and Mello in the year 1998 for which they were awarded Nobel Prize in 2006. The central role in this process is mediated by two types of small RNA molecules miRNA and siRNA. These double stranded molecules trigger suppression of gene activity in a homology-dependent manner. This phenomenon is seen in many eukaryotes and is initiated by an endonuclease DICER. The natural functions of RNAi have been found to be mainly in cellular defense against viruses, genomic containment of retrotransposons, and post-transcriptional regulation of gene expression. Infact, RNAi is proving to be of immense importance in the present scenario for treatment of various diseases in plants/animals, drug designing, crop improvement, increasing the quality of food, pest/weed control and will most likely lead to novel medical applications in the future.

**COMPONENTS OF RNAi MACHINERY**

Analysis of mutants defective in RNAi revealed a number of proteins and enzymes essential to this process. Some of the components identified serve as initiators and others as effectors, amplifiers and

transmitters of gene silencing process.

### Dicer

The enzyme was first discovered in *Drosophila* (2). It belongs to the RNase III-family, which show specificity for dsRNA and cleave them with 3' overhangs of 2 to 3 nucleotides and 5'-phosphate and 3'-hydroxyl termini (3). They can cleave long dsRNAs and stem-loop precursors into siRNAs and miRNAs in an ATP-dependent manner, respectively (4). Dicer contains several characteristic domains, a N-terminal helicase domain, PAZ domain (found in Piwi/Argonaute/Zwille proteins), dual RNase III domains, and a double stranded RNA-binding domain (2). Animals generally encode a single type of dicer with exceptions of *Drosophila* and *C. elegans* which encode two dicers (5). Unlike animals, plants usually encode multiple dicers. In *Arabidopsis*, DCL 2, 3, 4 are involved in the generation of different siRNA species while DCL1 solely helps in miRNA synthesis. Each dicer produces siRNAs of characteristic length, e.g. DCL2, DCL3 and DCL4 generate 22, 24 and 21 nt long siRNA species, respectively (6-8).

### RNA-induced silencing complex (RISC) and Argonaute

RNA interference is initiated in cell's cytoplasm by short double stranded RNA molecules where they interact with RISC located in ribosome. The RISC proteins have been identified by mass spectrometry as Argonaute 2, (9) VIG, Dfxr (10) and TSN. The duplex siRNAs are unwound by helicase activity of Argonaute. Argonaute has three domains, PAZ (near N-terminal), MID and PIWI (near C-terminal) domains (11). Animals and plants encode multiple Argonaute homologs like AGO1, AGO2, AGO6 etc. (12). AGO6 is involved in DNA methylation and transcriptional gene silencing (13) while AGO7 has been shown to participate in both tasi-RNA and long siRNAs biogenesis (14).

### RNA Helicases

They are a diverse array of proteins specific for each organism seems to carry out aberrant RNA elimination surveillance in most eukaryotes. The *A. thaliana* mutant SDE3, defective in production of RNA helicase is unable to carry out RNAi process. The SMG-2 homolog from *C. elegans* have ATPase, RNA binding and helicase activities and contain conserved cysteine-rich region near the C-terminus.

### RNA-dependent RNA polymerase (RdRP)

The major function of this protein is to generate secondary siRNAs in a primer-dependent and independent fashion and in amplifying silencing effect. The RdRP enzymes recognize the aberrant RNAs as templates and synthesize antisense RNAs to form

dsRNAs that are finally the targets for sequence-specific RNA degradation (15,16).

### Small interfering RNAs (siRNAs)

It was in 1999 when Hamilton and Baulcombe reported the presence of 21-25 nt. long dsRNA complimentary to both strands of silenced genes in *Arabidopsis* undergoing PTGS called siRNA's. They contain two nucleotide 3'overhangs (17). These RNA were also seen to silence expression in naïve *Drosophila* S2 cells and embryo extracts (17,18).

### Four types of siRNAs have been identified depending on the biogenesis of dsRNA precursor and its source.

- **Trans-acting short interfering RNAs (tasiRNA):** They are ~21 nt. long and closely related to miRNAs in size and function. They require endogenous transcript as template, (19,20) and RdRP, AGO-7 and DCL-4 for their production from template transcript (21). Organisms which lack RdRP like humans and flies, are devoid of tasiRNAs.
- **Repeat-associated short interfering RNAs (rasiRNAs):** These are ~24-26 nts. long and require DCL3 and RdRP for their synthesis. They function in gametogenesis in flies and in silencing of viral transcripts.
- **Scan RNAs (scnRNAs):** They are much longer, ~29 nts. long. It was first discovered in a protozoan *Tetrahymena thermophila*, where it produced modified newer versions genome from the existing ones (22).
- **Long siRNAs (lsiRNAs):** They are ~30-40 nt. long and require AGO-7, DCL-1 and DCL-4 for their biogenesis. They cause decapping of target transcripts and finally their degradation via. an exonuclease (XRN4).

The target specificity of siRNA require a "seed region" which occurs at the 5' end of siRNA encompassing ribonucleotides between 2 to 7 position and confer target specificity to siRNA. The siRNA containing a single mismatch with their substrate fail to repress the target mRNA and don't simply shift their regulatory mode to translation inhibition.

### Micro-RNAs (miRNAs)

The miRNA were discovered in 1993. Lee and colleagues in 2004 elucidated the function of a non-coding transcript in *C. elegans*, whose expression varied spatio-temporally and the mutants showed developmental abnormalities (5). MicroRNA genes

constitute ~1% of the total coding genes and form the largest class of regulatory molecules (23,24). They show high tissue-specific and temporal expression and are believed to have evolved to take intensive care of developmental pathways that can be achieved by translation suppression (occurring mainly in animals) or target cleavage (occurring mainly in plants) (25,26). They have been named variously i.e., miRNAs which mediate spatial development are referred to as sdRNAs, while cell cycle miRNAs are referred to as ccRNAs, etc. Their biogenesis is believed to be operative by more than one pathway, as described below:

**1. Canonical miRNA pathway:** Micro-RNAs are ~19-23 nucleotide long single-stranded RNAs. Prior to maturity, they first undergo extensive post-transcriptional modification. They are expressed from a much longer RNA-coding primary transcript known as a pri-miRNA which is processed, in the cell nucleus, to a 70-nucleotide stem-loop structure called a pre-miRNA by the microprocessor complex. This complex consists of an RNase III enzyme called Drosha and a dsRNA-binding protein DGCR8. The dsRNA portion of this pre-miRNA is bound and cleaved by Dicer to produce the mature miRNA molecule that can be integrated into the RISC complex. Maturation of miRNA begins in the nucleus and terminates in the cytoplasm.

**3.6.2 Mirtrons:** Animals have been shown to follow yet another mode of miRNA biogenesis where intron sequences can produce miRNAs. These miRNAs originating from introns were termed mirtrons (27,28). Fourteen mirtrons from *Drosophila* and four mirtrons from *C. elegans* have been identified so far. Mirtrons have not yet been identified in plants and other organisms.

The miRNAs are thought to direct RISC-related complexes to their targets on the basis of sequence complementarity. In contrast to this, most animal miRNAs appear to recognize their targets by imperfect pairing. For example, the interaction between *let-7* and its target *lin-14* involves multiple binding sites, each of which pairs imperfectly with the small RNA. This problem does not exist in plants where almost all miRNAs are extensively complementary to their targets (29,30) and act as siRNAs to direct endonucleolytic cleavage of target mRNAs (29,31,32).

Animal miRNA, as a general rule, binds to the 3' UTR region of the target (33,34) while in case of plants, binding occurs in the coding region (35). The seed region determines the target sequence and helps in miRNA editing (36). In animals, studies suggest that miRNA binding promotes either deadenylation or decapping of the target which is probably achieved by interaction of

RISC associated proteins with cap or poly-A tail associated proteins (25,37). miRNA-repressed transcripts in animals are engulfed into dynamic vesicles called P (Processing)-bodies (GW1 or cytoplasmic bodies) that carry out active mRNA degradation via nonsense mediated decay and gene silencing (38,39). Target mRNA, via interaction between one of the RISC members (AGO1) and the P-body proteins (GW1 and AIN1) gain entry into these structures (40). These bodies act as storage sites for translationally suppressed mRNA that are released when required and can actively translate (41). P-bodies have been shown to be associated with various components of translation machinery (except ribosomes) and RNAi components like AGO1, GW182, miRNAs and CCR4 (41). These are yet to be discovered in plants but some similar nuclear foci called Cajal bodies have recently been reported from *Arabidopsis* (42).

### GENERAL MECHANISM

The process of RNA interference can be divided into four stages, which are as follows:

**1. Double stranded RNA cleavage:** The first step includes ATP-dependent, processive dsRNA cleavage into double-stranded fragments 21 to 25 nucleotides long containing 5' phosphate and 3' hydroxyl termini, and two additional overhanging nucleotides on their 3' ends (17,43). The length of the cleaved RNA maximizes target-gene specificity and minimizes non-specific effects. Exogenous dsRNA is detected and bound by an effector protein, known as RDE-4 in *C. elegans* and R2D2 in *Drosophila* that stimulates dicer activity. In *C. elegans*, this initiation response is amplified through the synthesis of a population of 'secondary' siRNAs by an RNA-dependent RNA polymerase (RdRP) during which the dicer-produced initiating or 'primary' siRNAs are used as templates (Fig.1)

**2. Activation of silencing complex:** In the second step siRNAs are incorporated into RISC (RNA-induced silencing complex) which undergoes activation in the presence of ATP so that the antisense component of the unwound siRNA becomes exposed and allows the RISC to perform the downstream RNAi reaction. This siRNA duplex containing ribonucleoprotein complex is called siRNP (44). This formation requires ATP which reflects energy need for unwinding step and other conformational requirements. Members of Argonaute family probably help in stabilization of siRNA, RISC formation and mRNA targeting. siRNA, RISC formation and mRNA targeting.

**3. Unwinding of the siRNA duplex:** The third step



involves unwinding of the siRNA duplex and remodelling of the complex to create an active form of RISC. Only one of the two strands, which is known as the guide strand, binds the argonaute protein and directs gene silencing. The other anti-guide strand or passenger strand is degraded. This process is ATP-independent and performed directly by the protein components of RISC. The strand selected as the guide tends to be the one whose 5' end is less stably paired to its complement. The R2D2 protein may serve as the differentiating factor by binding the more-stable 5' end of the passenger strand. The phosphorylated 5' end of the RNA strand enters a conserved basic surface pocket and makes contacts through a divalent cation and by aromatic stacking between the 5' nucleotide in the siRNA and a conserved tyrosine residue.

**4. Cleavage of mRNA:** The final step includes the recognition and cleavage of mRNA complementary to the siRNA strand present in RISC (guide strand of siRNA), (44,45) by exoribonucleases. In some organisms (*C. elegans*, *Arabidopsis thaliana*) an additional step in the RNAi pathway has been described involving a population of secondary siRNAs derived from the action of a cellular RNA-dependent RNA polymerase (RdRp). Eukaryotic cells possess two major means for regulating the turnover of mRNAs. In one pathway they utilize the 3'-5' exonuclease, exosome, to degrade the message in the cytoplasm (46). The other pathway is thought to occur in specialized centers known as processing (P) bodies/cytoplasmic bodies/mRNA decay centers (38,39). These centers contain decapping enzymes Dcp1 and 2 and the 5'-3' exonucleases Xrn1(39). It is thought that transcripts are transported to these centers to be degraded by the 5'-3' exonuclease, Xrn1. Ago2 was found to be localized to the cytoplasm, with most of the Ago2 concentrated in discrete cytoplasmic bodies, the mammalian equivalent of yeast P-bodies (25,40).

## FUNCTIONAL ASPECTS AND APPLICATIONS OF RNAi

### Defence

RNA interference acts as a defense mechanism against viruses and other foreign genetic material, especially in plants. It may also prevent the self-propagation of transposons. Apart from plants there is significant debate over the ability of siRNAs and longer dsRNAs to induce innate immune response (47,48). In mammalian cells molecules less than 30 bp in length are generally believed to avoid induction of interferon pathways (47-49). Long (27-29 bp) dsRNAs and shRNAs provide more efficient gene silencing than shorter, Dicer-independent substrates (48,49). Some plant genomes also express endogenous siRNAs in response to infection by specific types of bacteria. These effects may be part of a

generalized response to pathogens that downregulates any metabolic processes in the host that aid the infection process. In both juvenile and adult *Drosophila*, RNA interference is important in antiviral innate immunity and is active against pathogens such as *Drosophila* X virus. A similar role in immunity may operate in *C. elegans*, as argonaute proteins are upregulated in response to viruses and worms that over express components of the RNAi pathway are resistant to viral infection.

### Down and up-regulation of genes

Endogenously expressed miRNAs, including both intronic and intergenic miRNAs, are most important in translational repression. The roles of endogenously expressed miRNA in downregulating gene expression were first described in *C. elegans* in 1993. In plants, the majority of genes regulated by miRNAs are transcription factors thus miRNA activity is particularly wide-ranging and regulates entire gene networks during development by modulating the expression of key regulatory genes, including transcription factors as well as F-box proteins. RNA sequences (siRNA and miRNA) that are complementary to parts of a promoter can increase gene transcription, a phenomenon dubbed RNA activation. Here, dicer and argonaute play major role, possibly via histone demethylation. They have also been proposed to upregulate their target genes upon cell cycle arrest.

### Development

Mutations in dcr-1 and ego-1 lead to complete sterility, (50,52) indicating importance of RNAi in germline development. In plants also RNAi-like processes have a crucial role in development (53,54). It is seen to be involved in maturation of endogenously encoded miRNAs, (51,55) some of which are involved in development, but most of which have no known function at present.

### Gene function analysis

Double-stranded RNA is artificially synthesized, complementary to a gene of interest and introduced into a cell where it is recognized as exogenous genetic material and activates the RNAi pathway. Using this mechanism, researchers can cause a drastic decrease in the expression of a targeted gene which can show the physiological role of the gene product. Functional analysis of almost all the ~19,000 genes of *C. elegans* has been carried out with the siRNA-directed knockdown approach (56).

### To determine gene function

- RNAi is sequence-specific and thus can be targeted, requiring only a few transformants per target gene.
- RNAi is dominant, so phenotypes can be observed in the T1 generation.
- RNAi often leads to partial knockdown and thus to a range of phenotypes of differing severity; this facilitates the study of essential genes whose inactivation would lead to lethality or extremely severe pleiotropic phenotypes.
- RNAi can be controlled in a tissue-specific or time-dependent manner RNAi can be quickly and easily used in a wide range of genotypes or even species, whereas insertion mutant collections are limited to just a few due to the effort involved.
- RNAi can be used to reduce the expression of several related genes in parallel by targeting conserved regions of the genes, facilitating the study of redundant gene functions.

### Heterochromatin formation

There is derepression of the centromeric outer-transposon repeats in RNAi mutants deficient for RNAi components. This led to the proposal that small RNAs function as guides to target the chromatin modifications that are typical of heterochromatin (57). There is reduced amounts of H3K9 methylation and repeat-associated Swi6 in the RNAi mutants. Second evidence was that a transgene that is located in the centromeric repeats, which would usually be silenced, was activated in RNAi mutants. Apart from centromeric heterochromatin formation, RNAi pathway is also implicated in the targeting of non-centromeric, interstitial sites in euchromatin for silencing. In *D. melanogaster*, dispersed transgenes that are inserted at several sites in euchromatin are silenced at the transcriptional level through association with the POLYCOMB COMPLEX(58) through histone modifications which is dependent on components of RNAi pathway. The Polycomb-dependent silencing involves histone modifications and is known to keep the chromatin in the closed or compact conformation. Heterochromatin formation requires that histone H3 of the chromatin is first deacetylated and then methylated at lysine 9. This methylated lysine is subsequently bound by a heterochromatin binding protein, HP1 in highly specific manner and with a very high affinity (59).

### RNA INTERFERENCE AS A THERAPEUTIC TOOL

#### In Animals

RNAi is having a lot of applications in biomedical research and health care and has begun to produce a paradigm shift in the process of drug discovery (60). Presently, many dsRNA molecules are being designed for silencing specific genes in humans and animals as shown in Table 1. It may be possible to exploit RNA interference in therapy. Although it is difficult to introduce long dsRNA strands into mammalian cells due to the interferon response, the use of ds RNAs shorter than ~30 nts are proving to be successful. The very first application of RNAi were mainly in the treatment of macular degeneration and respiratory syncytial virus knockdown of host receptors and coreceptors for HIV, the silencing of hepatitis A and hepatitis B genes, silencing of influenza gene expression, and inhibition of measles viral replication. Potential treatments for neurodegenerative diseases have also been proposed, with particular attention being paid to the polyglutamine diseases such as Huntington's disease. Despite the proliferation of promising cell culture studies for RNAi-based drugs, some concern has been raised regarding the safety of RNA interference, especially the potential for "off-target" effects in which a gene with a coincidentally similar sequence to the targeted gene is also repressed. A computational genomics study estimated that the error rate of off-target interactions is about 10%.

#### In Plants

This RNAi technology has proven to be an eco-friendly technique for crop improvement. Through this technique the genes which are responsible for inducing various stresses in plants are silenced and novel traits like disease resistance are incorporated into plants. It has emerged as a method of choice for gene targeting in fungi, (61) viruses, (62,63) bacteria (64) and plants (65). It has been used for applications in biotechnology, particularly in the engineering of food plants that produce lower levels of natural plant toxins as shown in Table 2. Other plant traits that have been engineered in the laboratory include the production of non-narcotic natural products by the opium poppy, resistance to common plant viruses, and fortification of plants such as tomatoes with dietary antioxidants.

### DISADVANTAGES OF RNA INTERFERENCE

#### Off-target effect

In large-scale screens in animals it was observed that the silencing effects were also seen in genes that were

not the predicted targets of RNAi. The major difficulty is the limited sequence specificity of siRNAs, as few as seven nucleotides of sequence complementarity between a siRNA and an mRNA can lead to the inhibition of expression.

A second specificity problem can occur via 'transitive silencing', whereby RNAi against a gene-specific sequence 'spreads' into neighbouring sequences conserved between the target mRNA and mRNAs from related genes, which become silenced in turn.

### Inefficacy and instability

RNAi inhibition can have widely varying effects depending on the target gene, the region of the transcript that is targeted and even between sibling plants carrying identical RNAi constructs. The instability can result from silencing of the transgene long hairpin transgenes appear to be particularly sensitive to transcriptional silencing leading to a loss of RNAi phenotypes over several generations.

### Conclusion and Future Prospects

RNA interference is an area of intense, upfront basic research and holds the key to various technological applications in future due to their higher silencing efficiency and shorter time requirements for screening and to analyses functions of wide variety of genes in different organisms. The RNA silencing technology apart from being highly sequence specific is also technologically facile and economical. Therefore, this technique has great potential in agriculture specifically for nutritional improvement of plants and the management of various plant diseases. Future directions will focus on developing finely tuned RNAi-based gene

silencing vectors that are able to operate in a temporally and spatially controlled manner. In coming years better and comprehensive understanding of RNAi would allow the researchers to work effectively and efficiently in order to work more on improvement of crop plants nutritionally and in managing various diseases of crop plants. Finally, the discovery of RNAi has not only provided us with a powerful new experimental tool to study the function of genes but also raises expectations about future applications of RNAi in medicine.

### CONFLICT OF INTEREST

The authors declare that they have no competing interests.

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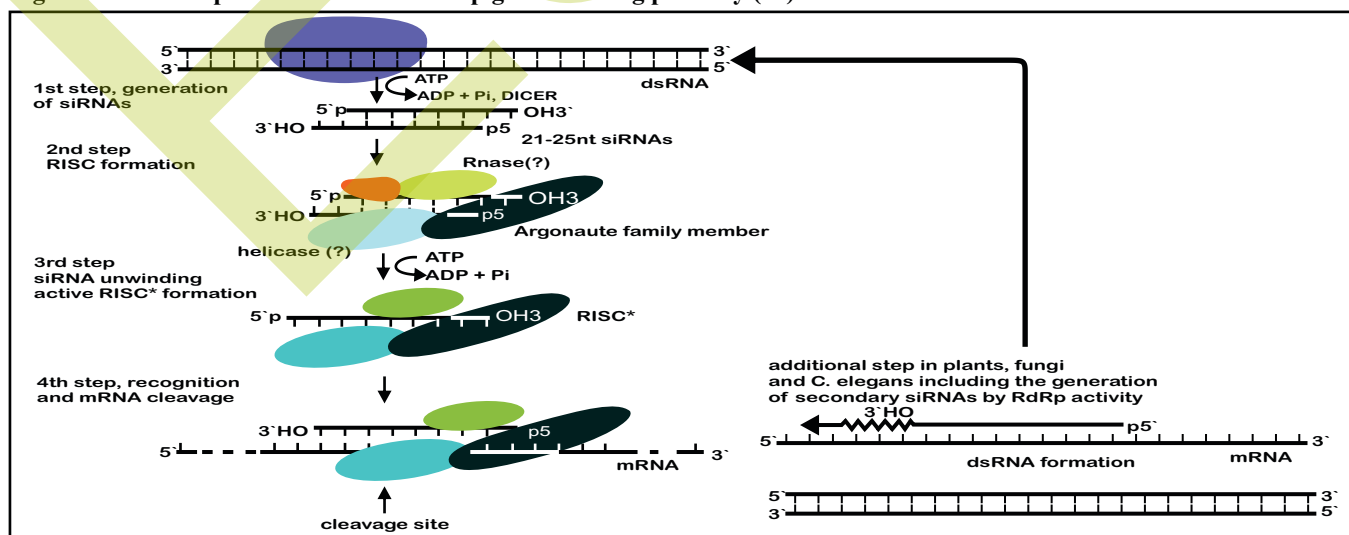
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**Table1. Application of RNAi in treating human disease (66)**

Disease	Case study
Lymphoblastic leukemia	Using siRNA's specific for the BCR - ABL transcript to silence the oncogenes
Bladder cancer	Treatment by miRNA's as biomarkers
HIV	Downregulation of cellular cofactors required for HIV infection by RNAi
Viral hepatitis	Inhibition of Fas expression by siRNA
Ocular diseases	Shutting down production of VEGF by siRNA
Metabolic disease and neurodegenerative disorders	Treatment of these diseases by miRNA's as potential therapeutic targets.
Cardiovascular and cerebrovascular diseases	Used to reduce damage to heart tissues and brain cells
Pancreatic and colon carcinomas	Use of retroviral vectors to introduce interfering RNAs specific for an oncogenic variant of K -Ras

**Table 2. Application of RNAi in plants (67)**

Application	Case study
Increasing the level of lysine	Reduction of lysine catabolism and improving seed germination generating a dominant high-lysine maize variant by knocking out the expression of the 22-kD maize zein storage protein
Barley and Rice	Resistance of barley to BYDV and producing a rice variety called LGC-1 (low glutenin content 1)
Banana	Production of banana varieties resistant to the Banana Bract Mosaic Virus (BBRMV)
Cotton	Transgenic cotton plants expressing a RNAi construct of the d-cadinene synthase gene of gossypol synthesis fused to a seed-specific promoter caused seed-specific reduction of Gossypol
Wood and fruit quality	-Down regulation of lignin biosynthesis pathways. -Producing transgenic hypoallergenic apples and a possible solution for the undesirable separation of juice into clear serum and particulate phase
Coffee	RNAi technology has enabled the creation of varieties of Coffee that produces natural coffee with low or very low caffeine content
Healthier oil	Using RNAi to silence the gene in cotton which codes for the enzyme that converts oleic acid into a different fatty acid
Tomato	RNAi-mediated suppression of DET1 expression under fruit-specific promoters has recently shown to improve carotenoid and flavonoid levels in tomato fruits with minimal effects on plant growth
Gentian	Producing white-flowered transgenic gentians by suppressing the chalcone synthase ( <i>CHS</i> ) gene .
Blue Rose	Producing blue transgenic rose by knock down the cyanidin genes in rose and carnation .
Pest control	Combining Bt technology with RNAi would both enhance product performance and further guard against the development of resistance to Bt proteins

**Fig1. Schematic representation of four-step gene silencing pathway (68)**



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